

Development of Giant Motor Axons and Neural Control of Escape Responses in Squid Embryos and Hatchlings

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Abstract. Anatomical development of the third-order giant axons was studied in conjunction with ontogeny of the escape response and the underlying neural control. Stimulated escape jetting appears at stage 26 (Segawa *et al.*, 1988); such responses are driven solely by a small axon motor system. Giant axons become morphologically identifiable in the more posterior stellar nerves that effect jetting by stage 28, and electrical activity in the stellate ganglia associated with the giant axons is first recordable at this time. Maturation of the giant axons is accompanied by a marked improvement in temporal aspects of escape behavior up to the time of hatching. In embryonic and hatchling *Loligo*, all escape responses, regardless of the mode of stimulation, are fast-start responses with latencies less than the minimum value displayed by adults (50 ms). Giant axon activity recorded in the stellate ganglion always precedes small axon motor activity; this is not true for adults which display two distinct modes of giant axon use. Both giant and non-giant motor systems are thus functional in embryonic and hatchling squid, and both contribute to escape jetting. However, these animals do not yet display the concerted interplay of the two motor systems characteristic of adults.

Introduction

Lolliginid squid possess giant neurons with very large axons that are important components of the motor pathways mediating jet-propelled escape responses (Young, 1938). Anatomical details of the giant fiber pathway were beautifully described at the light microscope level over 50 years ago by Young (1939). In brief, two bilaterally sym-

metrical first order interneurons lie in the magnocellular lobe of the brain and receive massive sensory inputs from many sources, including the statocysts, optic lobes, and mechanoreceptors in the tentacles. These cells are unusual in that their short axons are fused via a cytoplasmic bridge. Each first-order giant axon contacts seven second-order giant cells in the palliovisceral lobe. The largest of these cells are interneurons, and each projects a giant axon via the pallial nerve to the ipsilateral stellate ganglion where it contacts the third-order giant motor axons of the stellar nerves. The six other second-order giant cells are motoneurons that innervate the musculature associated with head retraction, siphon aiming, and ink ejection.

This basic plan is straightforward, but oversimplified. Each of the above giant cells receives numerous other synaptic inputs, about which little is known (Boyle, 1986). The first-order giants also receive major inputs from higher-order centers such as the cerebral ganglia. Integrated outputs from these regions must influence activity of the first-order giants, which are likely to be an important decision-making element, given the commanding anatomical position they hold. Complexity in the giant fiber pathway and the biological necessity to strictly control giant fiber excitation was clearly recognized by Young (1939), who pointed out that lack of such control “. . . would lead to behavior by the squid even more ‘nervous’ than that for which the animals have a reputation.”

Despite the wealth of anatomical data, physiological studies of squid escape behavior have lagged far behind work on many other preparations (Eaton, 1984; Mackie, 1990). The pioneering work of Young [1938; Prosser and Young (1937)], in which reflex activation of escape jetting was inferred from studies of nerve-mantle preparations, was followed up only much later by Wilson (1960), who studied control of mantle contractions by a small axon

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system as well as by the giant axons. Since that time it has become widely accepted that (1) high-pressure escape jetting is mediated solely by the giant axon pathway essentially as a reflex and that (2) low-pressure respiratory 'jetting' is the primary, if not only, function of the small axon (non-giant) motor system.

Reinvestigation of these ideas has revealed a more complex picture (Otis and Gilly, 1990). Recordings of stellar nerve activity during escape responses *in vivo* show that strong escape jets can be driven by the small axon system acting independently or in concert with the giant axons. Moreover, the giant axon pathway can be used in two distinct modes. One produces a short latency startle response, whereas the second leads to a complex delayed escape response. In the latter case, critically timed excitation of the giants provides a potent, but secondary boost to the jet.

The present study investigates the neural control of escape jetting in embryonic and hatchling squid. All three neural elements of the giant fiber pathway are highly developed at the time of hatching (Young, 1939), and escape jetting capabilities are respectable (Packard, 1969). Embryonic development of the first-order giant neurons has been studied in detail (Martin, 1965, 1969, 1977), but much less is known about the second- and third-order giants (Marthy, 1987). Behavioral and neurophysiological studies concerning development of the giant fiber pathway are completely lacking.

This paper describes the ontogeny of the escape response in relation to development of the giant fiber system, with emphasis on the role of the third-order motor axons. Several questions are addressed, answers to which provide the framework for future studies. When do the giant motor axons develop morphologically, and when do they begin to mediate escape responses? What functional consequences are manifested in parallel with growth of the giant axons? How does the pattern of giant axons use compare to that in the adult animal? Finally, when does controlled interplay between the giant and non-giant motor systems develop?

Materials and Methods

Animals

Loligo opalescens was collected from Monterey Bay and maintained in holding tanks plumbed with flow-through seawater ($\sim 13^{\circ}\text{C}$). Spawning occurred in these tanks, and clusters of fertilized eggs were maintained in small mesh enclosures in gently flowing seawater until natural hatching occurred (~ 30 days). Staging of animals was carried out following criteria of Segawa *et al.* (1988) with only minor modifications (see Results and Table I).

Septoteuthis lessoniana was supplied by the Marine Biomedical Institute, University of Texas Medical Branch, Galveston, where the animals had been reared. Animals

ranging from 5 to 21 days post-hatching were shipped overnight and studied the following day.

Behavioral experiments

Loligo from stage 24 through post-hatching was used for studying development of escape-jetting behavior. To obtain animals for each experiment, a "finger" of eggs was disrupted, the eggs were dispersed in seawater, and the chorionic membranes were ruptured to release the embryos. Individual embryos were chosen from this pool and staged under a stereomicroscope.

After staging, an animal was placed in a 35 mm culture dish lined with Sylgard (Dow-Corning, Midland, Michigan) and filled with seawater at room temperature ($16\text{--}20^{\circ}\text{C}$). The animal was lightly restrained (ventral surface up) with a wire yoke, which was formed to fit over the base of the arms and inserted into the Sylgard. Electrical stimuli ($20\text{--}60$ V, 6 ms duration) were delivered via a seawater-filled micropipette positioned near the ventral midline in the area of the brachial ganglion (see Fig. 2A).

Behavioral responses were recorded on conventional videotape. The stimulating pulse also triggered a small light source beneath the experimental chamber, which served as a timing marker. Although the exact frame when the stimulus occurred is identifiable with this method, stimuli were not synchronized with the video framing and uncertainty therefore exists about precisely when in the frame the pulse occurred (each video frame spans 33 ms). Data were later sampled in blocks of 16 real-time frames with an image-analysis system (Megavision 1024 XM, Santa Barbara, California) and analyzed on a frame-by-frame basis to determine the time course of the change in mantle diameter following stimulation (see Fig. 2B).

Electrophysiological experiments: adult squid

Adult specimens of *Loligo* were lightly anesthetized in 0.4% urethane in aerated seawater at 15°C . The mantle was slit ventrally along the midline, exposing the gills, and the squid was pinned ventral side up through the outspread mantle flaps to the bottom of a Sylgard-filled glass dish. A pin through the region of the mouth prevented head retraction. The left pallial nerve was cut proximal to the stellate ganglion, thereby immobilizing the mantle musculature on that side. On the right side, the stellate ganglion was exposed by removing the thin layer of skin that covers it. The larger, more posterior stellar nerves were severed close to their emergence from the ganglion, but the pallial nerve was left intact. This paralyzed the right side of the animal but retained synaptic transmission at the giant synapses and motor outputs in the stellar nerve stumps. Throughout these operations, chilled seawater was kept flowing over the gills. Upon completion of surgery, the urethane-seawater was ex-

changed for O₂-saturated seawater (12–15°C) which was thereafter perfused continuously over the gills.

Conventional extracellular recording techniques were used. One polyethylene suction electrode was attached to the pallial nerve for *en passant* recording. A second electrode was used to record from a stellar nerve by sucking the proximal stump into the lumen of the electrode. Voltage was measured differentially between Ag:AgCl wires, one in the electrode's lumen and the other wrapped around the tip. Polarity of the recordings was arranged so that the initial phase of activity in the third-order giant axon was positive-going. AC-coupled amplifiers served to amplify and low-pass filter (3–10 KHz) the signals, which were displayed on a digital oscilloscope or recorded onto videotape via a digital audio processor (sampling at 44 KHz; Unitrade, Philadelphia, Pennsylvania) for subsequent analysis using a laboratory computer.

Electrophysiological experiments: embryonic and hatchling squid

Recording techniques were adapted from those used with the adult animals. Hatchlings and embryos (removed from their chorions and staged as in the behavioral experiments) were pinned out with fine cactus spines, ventral side up, after slitting the ventral mantle wall to allow access to the stellate ganglia. No nerves were cut, and recordings from the stellate ganglion were made with a suction electrode applied directly over the ganglion through the overlying tissue. Recordings from the magnocellular lobe (site of the first-order giant neurons) required removal of the skin and cartilaginous material just anterior to the statocysts, and the recording electrode was applied directly to the nervous tissue thereby exposed. It was also necessary to remove some of the mass of small cells, presumably undifferentiated neuroblasts (Martin, 1965), which overlie the first-order giant cells by sucking or blowing water jets through the electrode tip before attaching it.

For all neurophysiological recordings, electrical stimuli were delivered through a fine coaxial metal electrode, and a triggered strobe light was used for delivering photic stimuli. Shocks of 1–3 ms, 20–80 V were most effective in stimulating adults and 0.1–0.4 ms, 10–50 V in hatchlings and embryos. All experiments were carried out at 12–15°C in the case of *Loligo* and 21°C with *Septoteuthis*.

Electron microscopy

Staged embryos were fixed in a 0.965 osmolar solution of 1% glutaraldehyde, 0.1% tannic acid, 0.2 M sodium cacodylate and sucrose (pH 7.4) and post-fixed in 2% osmium tetroxide, 0.8% potassium ferrocyanide, and 0.2 M sodium cacodylate (pH 6.8). Fixed material was dehydrated in graded hexalene glycol and embedded in Spurr's resin. Animals were sectioned both perpendicular and oblique to the long body axis to obtain transverse sections

of the posterior stellar nerves. *En bloc* staining was carried out in saturated uranyl acetate at 60°C for 3 h. Thin sections were examined in a Philips 301 transmission electron microscope operating at 60 kV.

Results

Developmental timetable of giant motor axons and jetting behavior

Loligo opalescens develops embryologically in close correspondence to *L. forbesi*, and staging criteria for the latter species (Segawa *et al.*, 1988) can be directly applied. Stages 24 through hatching (30), representing approximately half of the total developmental period, are relevant to the present study, and selected characteristics applicable to our staging of *L. opalescens* are summarized in Table I. Based on our work with hundreds of embryos in this study, ontogeny of jetting behavior can be related to these anatomical stages with a high degree of confidence, and this information is included in Table I and covered in detail below.

Electron microscopic examination of conventionally fixed and embedded material reveals that axons in stellar nerves which can be labeled 'giant' (*i.e.*, distinctly larger than any other processes) first occur at stage 26, but only in the more anterior nerves emanating from the stellate ganglion (Fig. 1A). The more posterior stellar nerves do

Table I

Staging characteristics employed and development of escape behavior in embryonic Loligo opalescens. Developmental staging follows the criteria described by Segawa et al. (1988) for Loligo forbesi, except in our work we lumped stages 27 and 27+ (into 27) and 28 and 28+ (into 28)

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- | | |
|-------------|---|
| Stage 25: | 1. Spontaneous, symmetrical mantle contractions begin. |
| Stage 26: | 1. Eyes are brilliant red.
2. Ink sac is visible.
3. Chromatophores are on dorsal side of head.
4. Electrical and mechanical stimulation of escape response is possible. |
| Stage 27: | 1. Ink is barely visible in ink sac.
2. Eyes are dark, but not black.
3. Edge of primary lid covers half of optic vesicle. |
| Stage 28: | 1. Eye is completely covered by primary lid.
2. Mid-gut gland is visible.
3. Yellow chromatophores are on arms.
4. Head retraction occurs during escape responses. |
| Stage 29: | 1. Olfactory organ is visible as thickened disk.
2. Light flashes stimulate escape responses. |
| Stage 29+: | 1. External yolk sac is equal to arm length. |
| Stage 29++: | 1. Spontaneous vigorous jetting occurs. |
| Stage 30: | 1. Natural hatching occurs; no external yolk sac. |
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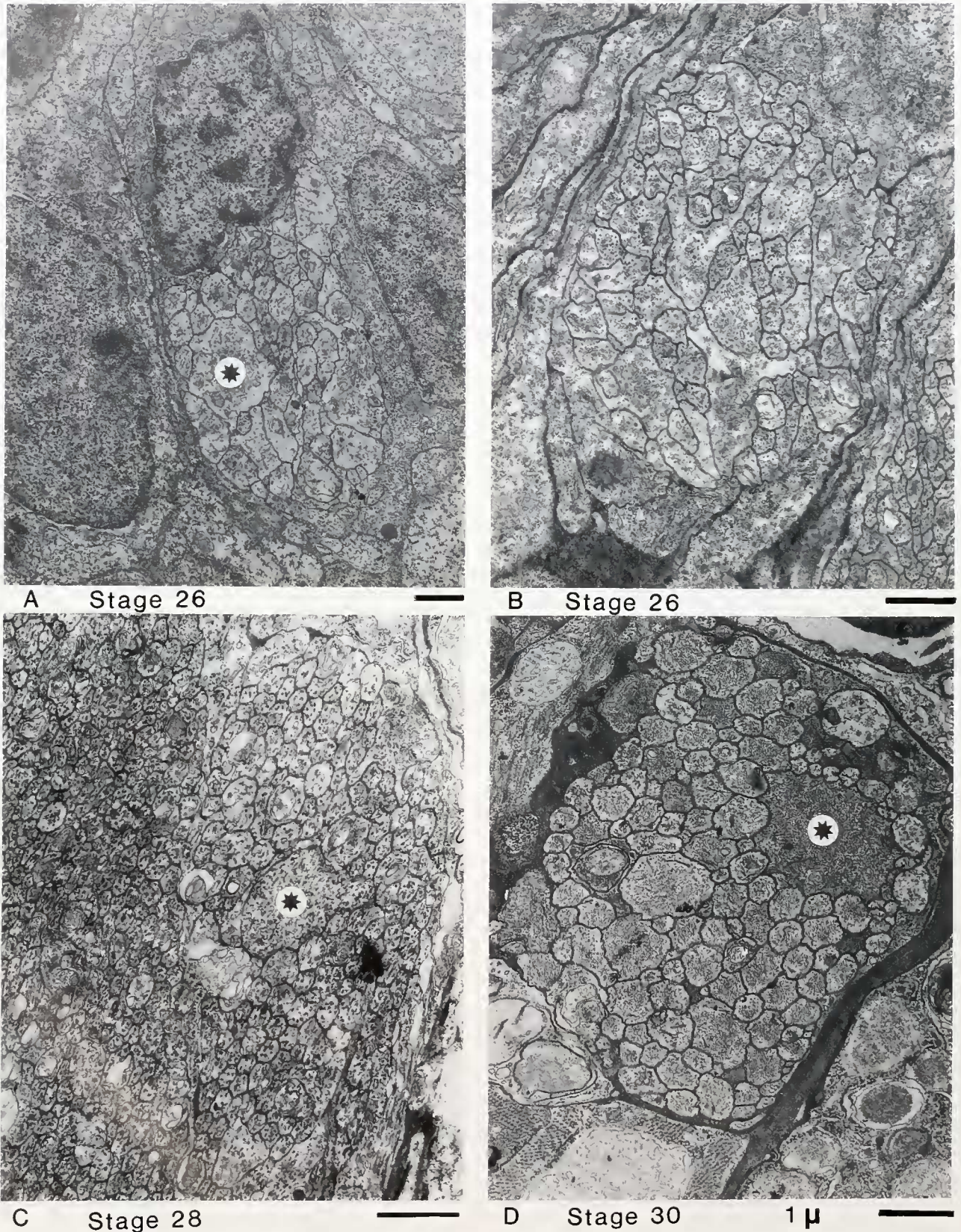


Figure 1. Anatomical development of the giant axons in stellar nerves of embryonic *Loligo opalescens*. Each panel shows a cross-section of a stellar nerve at the indicated developmental stage. (A) A single large axonal process is first identifiable in an anterior stellar nerve at stage 26 (*), although other processes are

not display a singularly large axon leading to the mantle muscle at this time (Fig. 1B), but clearly do so by stage 28 (Fig. 1C). As discussed below, stage 28 is the first time at which giant axon activity could be recorded from the stellate ganglion. Well-developed giant axons exist in all stellar nerves at the time of hatching, and an example of a hind-most nerve is shown in Figure 1D.

We have not characterized the apparent anterior to posterior wave of giant axon maturation in detail, nor do we at this time have a complete picture of where, when, and how the axons of giant fiber lobe motoneurons actually fuse to form the giant axons prior to stage 28. Giant processes can be identified in all stellar nerves at stage 27, but (in every case examined) only proximal to the first branch point of a stellar nerve shortly after it enters the mantle tissue.

Development of escape response: behavioral studies

Escape responses in embryonic and hatchling squid were stimulated with electrical shocks, strobe light flashes, or mechanical stimuli and videotaped for analysis. An example of such data from a stage 29 embryo is shown in Figure 2A. Sequential video frames, photographed from the display monitor of the image analysis system, are numbered -3 through 4. A brief electrical stimulus was applied during frame 0, and the timing is identifiable by a light flash marker (*). Mantle diameter is indicated by arrowheads in each panel, and Figure 2B illustrates the time course of mantle contraction (1 frame equals 33 ms). After a delay of one frame, mantle diameter decreases to 40% of its original (time 0) value over the subsequent four frames.

Peak response, delay, and time to peak (as indicated in Fig. 2B) were measured for electrically stimulated jets in 3-4 animals of every developmental stage from 25 through several days post-hatching. Mean data are summarized in Figures 3A-B, along with values obtained in similar experiments on adult animals in a previous study (Otis and Gilly, 1990).

Escape jetting increases in strength (Fig. 3A) smoothly up to hatching and then begins to slowly decline. Electrical stimuli (●) can elicit relatively strong responses at stages 26-27, a time when there is no anatomical sign of giant axons in the more posterior stellar nerves that innervate the mantle area in which diameter was monitored. Presumably these responses are mediated by the small motor axon system, which also can drive strong escape jets in adults. There is no dramatic sign of increased strength in

the response accompanying the appearance of functional posterior giant axons by stage 28. Mechanical stimulation (▲; taps with a fine probe) also leads to strong escape jets, and light flashes (○) become effective at stage 29.

A functional correlate of the development of the giant axons in the posterior stellar nerves is suggested in Figure 3B. Both time-to-peak response (●) and delay (■) decrease dramatically between stage 29 and hatching. As described below, giant axon activity can be first recorded in the stellate ganglion at stage 28, and the developmental decrease in behavioral delay (Fig. 3B) is also evident in the electrical recordings (Fig. 10A).

Development of giant axons thus improves temporal aspects of jetting performance. Acceleration of a squid through water is a function of both intra-mantle pressure and the rate of change in pressure (O'Dor, 1988). Rate of change in mantle diameter is thus an important determinant of escape performance. Figure 3C plots the maximum change in mantle diameter (data in Fig. 3A) divided by the frames to peak change (data in Fig. 3B) for each developmental stage. A sharp rise in the rate of mantle contraction occurs just before hatching, and performance then declines towards the adult level.

Recordings of motor activity in adult Loligo

In a previous study examining the functional role of the giant motor axons (third-order) in escape jetting (Otis and Gilly, 1990), adult squid were attached to a plastic support platform by their dorsal mantle surface but were otherwise free to make unrestrained respiratory and swimming movements. Under these relatively natural conditions, it was demonstrated that squid show apparently normal behavioral responses in regard to escape-jetting when compared with free-swimming animals in large tanks. The present study goes a step further and shows that similar neural responses can be obtained with dissected, pinned, and inverted animals.

Electrical stimulation directly over the magnocellular lobe in an adult squid leads to a large, short latency action potential in the pallial nerve (2) followed by a spike in an ipsilateral stellar nerve (3) as illustrated in Figure 4A. These 'directly' evoked events are assumed to result from direct activation of the first-order giant cells and represent the sequential firing of the second- and third-order giant axons, respectively, as described by Bryant (1959).

The electrode on the stellar nerve also picks up a small potential just before the third-order spike, and this pre-

almost as large. (B) No singularly large axon exists in the hind-most stellar nerve at stage 26. (C) By stage 28, a distinctly large 'giant' axon is present in the hind-most stellar nerve (*). The fin nerve lies to the left of the stellar nerve and is composed entirely of small axons. (D) The hind-most stellar nerve at the time of hatching shows a well-developed giant axon (*).

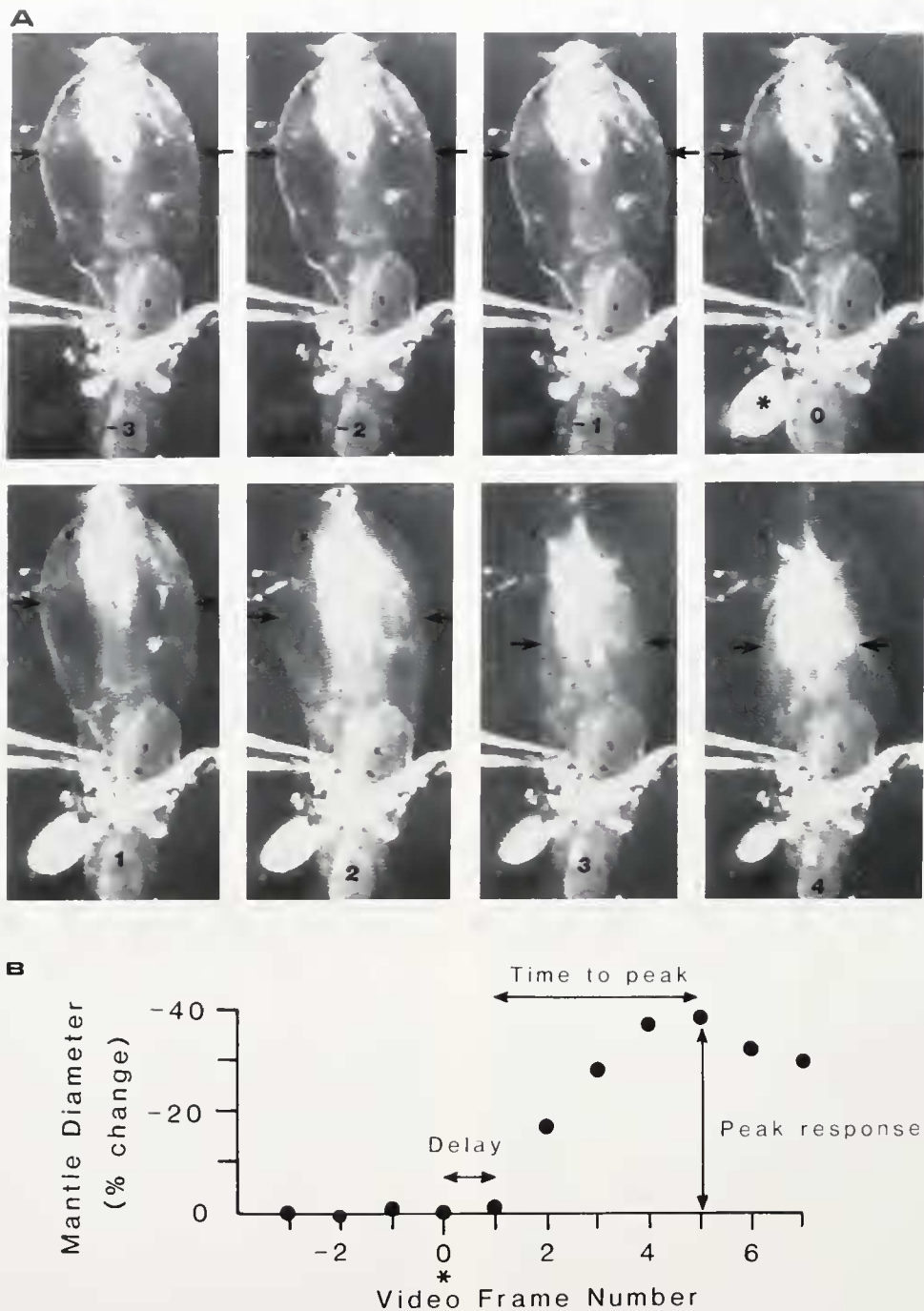


Figure 2. Escape behavior of a stage 29 *Loligo* embryo in response to an electric shock. (A) Sequential video frames are illustrated; the stimulus was applied via the pipette during frame 0 (*). Arrows indicate mantle diameter (measured at the widest point). See text for additional details. (B) Data from the experiment in Figure 2A is plotted in graphical form, and the parameters measured are indicated.

sumably represents the arrival of the second-order wave in the stellate ganglion. The delay between its peak and the start of the third-order event is approximately 1 ms and represents synaptic delay at the giant synapse. This characteristic short-latency pattern of activity in the stellate ganglion was consistently observed in adults (Fig. 4A),

hatchlings (Fig. 4B), and embryos (Fig. 6A) only when stimuli were applied directly over the magnocellular lobe. When the squid is stimulated by a light flash (Fig. 5A, B) or by electrical shocks in regions other than the immediate vicinity of the first-order giant cells, *e.g.*, the tentacles (Fig. 5C, D), 'indirect' responses are obtained. These occur after

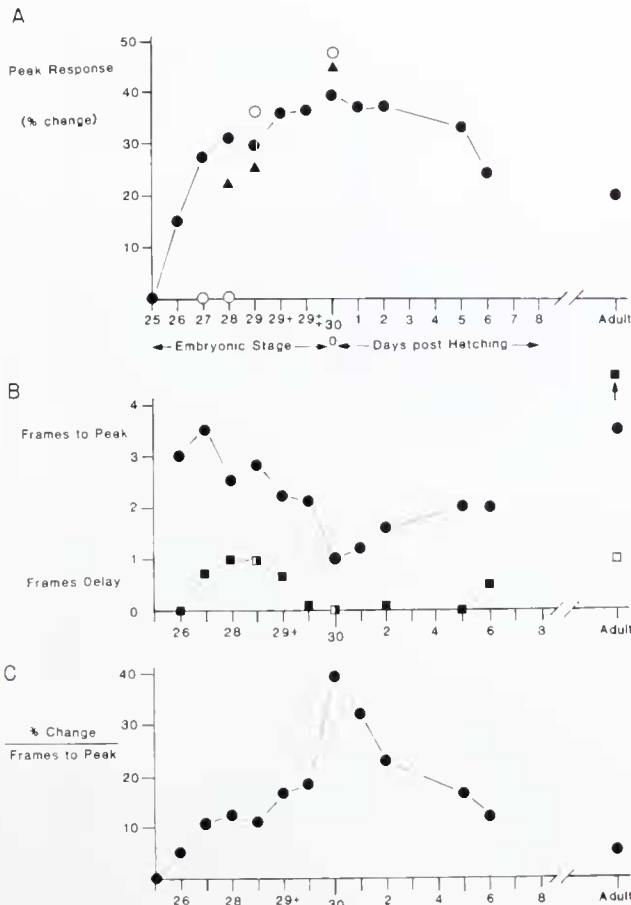


Figure 3. Ontogeny of escape jetting in *Loligo*. Three or four animals at each developmental stage were studied as described in conjunction with Figure 2, and mean values are plotted. Adult values are from data obtained in a previous study (Otis and Gilly, 1990). Electrical (●, ■), tactile (▲), and photic (○, □) stimuli are individually plotted. (A) Peak mantle contraction rises smoothly from stage 25 until hatching. Each of the stimulus modes yields strong escape jets. (B) Temporal aspects of escape performance [frames to peak (●) and delay (■, □)] improve markedly between stages 28 and 30. The adult value for delay with an electrical stimulus (■ over arrow) has a minimum value of 7–8 frames (Otis and Gilly, 1990). (C) Rate of mantle contraction, approximated as maximum diameter change divided by frames to peak, shows a sharp increase before hatching (stage 30) and a post-hatching decline towards the adult level.

sizable delays and represent activation through more physiological pathways.

General features of indirectly stimulated motor activity due to giant and non-giant (small) axon pathways observed in the present study closely resemble those previously reported for tethered, intact squid. In escape responses evoked by light flashes, the giant axons fire either after a 50 ms delay at the start of a burst of small axon activity (Figs. 5A, B) or not at all. In an indirect response stimulated by an electric shock, the giant axons fire only after a much longer delay (several hundred ms) and always during a burst of small-axon activity (Figs. 5C, D). Giant axons do not fire in every jet cycle, and the small-axon

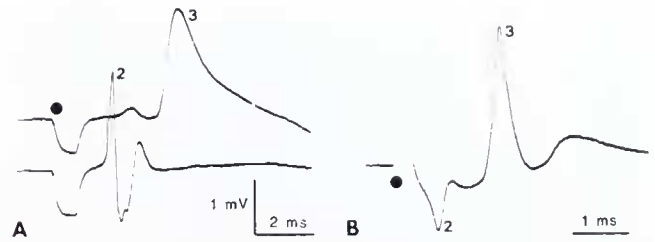


Figure 4. 'Direct' responses to electrical stimulation (●) applied over the magnocellular lobe in an adult *Loligo* (A) and a 7-day post-hatching *Sepioteuthis* (B). (A) The electrode on the pallial nerve (lower trace) records the second-order giant axon spike (2) *en passant*, while a second electrode on a posterior stellar nerve stump (upper trace) records the third-order spike (3) of the giant motor axon. (B) A single electrode placed over the stellate ganglion records both second- and third-order events. See text for additional details.

system acting alone can generate intense episodes of motor activity (second cycle in Fig. 5C). In all these respects our findings agree with Otis and Gilly (1990).

Recordings of motor outputs in hatchling and embryonic *Loligo*

Recordings from the stellate ganglion of late-stage embryos and hatchlings after electrical stimulation of the head show activity associated with both giant and non-

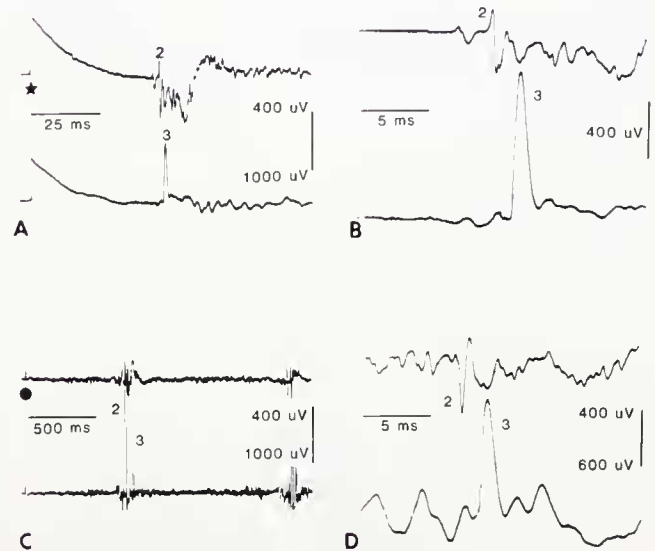


Figure 5. Bursts of motor activity associated with escape jetting in adult *Loligo*. In each panel (A–D) the upper trace is from the pallial nerve, and the lower trace is from a stellar nerve. (A) A light flash stimulus (*) results in firing of the giant axons (2 and 3) after a delay of ~50 ms and before the onset of the non-giant motor burst. (B) Portions of the records in (A) are displayed on an expanded time scale to illustrate the time course of the giant axon spikes. (C) An electrical stimulus (●) leads to giant axon activation after a long delay (>500 ms). In this case, the burst of small axon motor activity commences before the giant spike. A second escape cycle at the end of the record is driven by non-giant axons acting alone and shows no giant spike. (D) The first cycle in (C) is displayed at an expanded time base.

giant motor systems. Figure 6A illustrates such recordings from a stage 29+ embryo. Giant fiber excitation is indicated by an initial small, negative-going spike (2) followed by a larger positive event (3). As discussed above, the first component (2) represents arrival of the impulse in the second-order giant fiber entering the stellate ganglion via the pallial nerve, whereas the second component (3) reflects the summed action potentials from proximal parts of the third-order giants lying within the ganglion. Thus, moving the recording electrode anteriorly along the pallial nerve amplifies the second-order event and eliminates the third (Fig. 6B), whereas moving the electrode posteriorly along the larger stellar nerves isolates activity in the third-order giant fiber (Fig. 6C). Moving the electrode peripherally along one of the smaller (anterior) stellar nerves out into the muscle field shows the third-order spike followed by a large muscle potential (m, Fig. 6D).

Following stimulation over the brachial ganglion, activity can be recorded at all three stages in the giant fiber pathway by placing one recording electrode over the magnocellular lobe and a second on the stellate ganglion. The first electrode records the first-order giant spike (1) while the other records the second- and third-order events in sequence (Figs. 7A, B).

Firing of the first-order cell is invariably followed by activation of the other two elements, and the delay from the peak of the first-order spike to the start of the third-order event is only 2.0–2.5 ms. The delay that precedes firing of the first-order spike during an indirect response is much longer (*e.g.*, 26 ms in Fig. 7A or 13 ms in Fig.

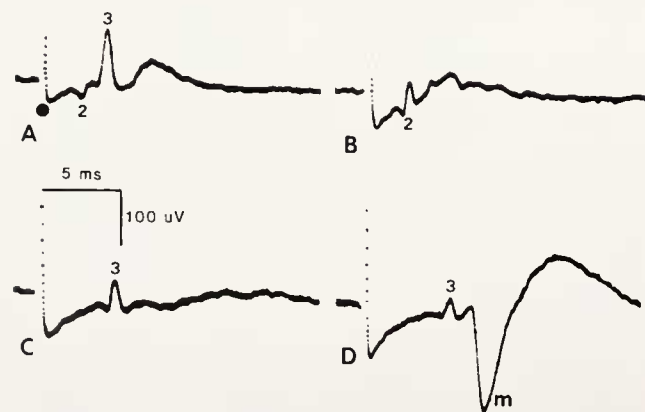


Figure 6. Electrical activity in the second- and third-order giant axons in a stage 29+ *Loligo* embryo. Direct responses of the giant fiber pathway were generated by stimulation over the magnocellular lobe. (A) The recording electrode was placed directly over the stellate ganglion, and the incoming second-order spike (2) and the out-going third-order wave (3) are recorded. (B) The electrode was positioned on the pallial nerve just proximal to the stellate ganglion, and the second-order spike is thereby isolated. (C) The electrode was placed on the emergence of the posterior stellar nerves from the ganglion: this isolates activity in the third-order giant axons. (D) The electrode was placed over an anterior stellar nerve in the muscle field of the mantle. This reveals the third-order giant axon spike and a large muscle potential (m).

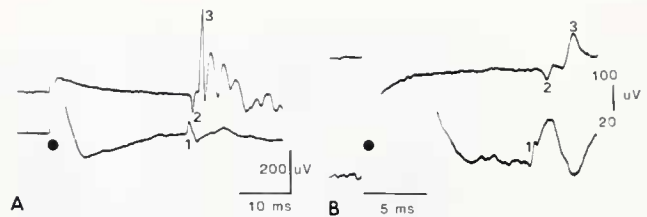


Figure 7. Timing of electrical activity from all three stages of the giant fiber pathway following indirect electrical stimulation over the brachial ganglion in a stage 29++ (A) or 30 (B) *Loligo*. In each panel the lower trace is recorded with an electrode on the magnocellular lobe, and a composite event including the first-order giant spike is obtained (1). Upper traces are recorded from the stellate ganglion, where second- (2) and third-order (3) events are detected.

7B) and presumably reflects 'processing' time in the central nervous system. Small potentials precede the initiation of the first-order spike in Figure 7B and must represent summed activity in pathways leading into the magnocellular lobe. There is also generally an outburst of small-unit activity coincident with and following the first-order giant spike, which itself appears difficult to resolve except for the rising phase (1 in Fig. 7B).

Indirectly stimulated escape responses in hatchlings produce the pattern of second- and third-order giant spikes discussed above (Figs. 8A, B), and the subsequent activity in the small motor axons takes the form of flurries of irregular, compound action potentials (Fig. 8A). Unit activity is generally difficult to resolve, and these potentials probably represent the firing of dozens of axons in rough synchrony. Although these compound events may approach third-order giant spikes in amplitude, they are readily distinguished from the latter by their irregular and variable waveforms and slower rise times. Bursts of small unit activity can be elicited by weak stimulation without excitation of the giant fiber system (upper trace in Fig. 8A) and would be associated in nature with non-giant escape jetting, as occurs in adults.

Two consistent and striking features characterize neural recordings of indirectly stimulated escape responses in late-stage embryos and hatchlings and clearly differentiate them from analogous records obtained in adults. First, the minimum latency for excitation of the third-order giant axons in hatchlings by photic stimulation is considerably shorter than that in adults (~ 15 ms in Figs. 8B, 9F vs. 50 ms for adults in Fig. 5A), and the latency following electrical stimulation in hatchlings can be nearly as brief (Fig. 8A). In adult animals, this latency is at least several hundred ms (Fig. 5C; *c.f.* Otis and Gilly, 1990).

A second and more pronounced difference is that when the third-order giants fire in hatchlings, their impulses arise in the stellate ganglion several ms before those of the small motor axons. This is true regardless of whether stimulation is via electric shocks (Fig. 8A) or light flash (Fig. 8B). This pattern of giant *versus* non-giant activity

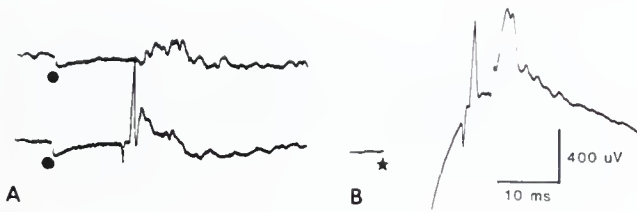


Figure 8. Comparison of motor outputs from a stage 30 *Loligo* in response to an electrical shock at the base of the tentacles (●, A) and to a light flash (*, B). (A) Upper trace shows activity of small axon system acting alone; stimulus was sub-threshold for giant fiber activation. Lower trace was recorded with a slightly stronger shock, which led to indirect excitation of the giant fiber pathway. The giant axon spike arrives in the stellate ganglion before the small axon activity. (B) A light flash produces a similar pattern of motor activity in which the small axon wave follows the giant fiber response.

for electrically stimulated responses is thus temporally inverted in comparison to the picture in adults, where giant axon spikes always fire 50–75 ms after the onset of the burst of non-giant activity (Fig. 5C, D; Otis and Gilly, 1990).

Development of motor patterns prior to hatching

Small axon control of jetting behavior is demonstrable at stage 26 (the earliest examined). Intermittent bouts of spontaneous rhythmic activity occur and represent normal respiratory cycles. Electrical stimulation evokes bursts of small unit activity resembling these respiratory bursts, but stimulated activity is generally more intense and long-lasting (Fig. 9A). Latency for small axon excitation appears to be brief at this stage, but this may reflect direct stimulation of these pathways in the small embryos at this early stage. Small axon responses continue with little change in the above pattern throughout subsequent developmental stages, except for an apparent increase in latency to 30–50 ms by stages 27–28 (Fig. 9C).

Light flashes are ineffective at triggering escape jets or neural activity in the stellar nerves at stages 26 (Fig. 9B) or 27 (not illustrated). Photic stimulation does produce bursts of small axon activity in the stellate ganglion by stage 28 (Fig. 9D), coincident with anatomical maturation of the eye (Segawa *et al.*, 1988).

Giant axon responses can first be evoked by electrical stimuli at stage 28 in embryonic squid (Fig. 9E) and by light flashes around the time of hatching (Fig. 9F). Generally, the picture of functional development of the giant fiber system from stage 28 through hatching is one of progressive maturation, in terms of speeding of the action potential waveform, reduced response latencies, and decreased synaptic delay. The immaturity of the giant fiber pathway at stages 28–29 is suggested by: (i) the small amplitudes and long durations of both second- and third-order giant spikes; (ii) the relatively long response latency—the second-order spike takes more than 20 ms to arrive in the stellate ganglion compared with a delay of

~12 ms at stages 29++ or 30 (Fig. 10A), and (iii) the progressive decrease for second- to third-order synaptic transmission during development (Fig. 10B). These changes parallel the improvement in behavioral performance as indicated by the square symbols and dashed curve in Figure 10A, which are behavioral data replotted from Figure 3B.

Post-hatching development of giant and non-giant motor patterns

Although the embryonic pattern of giant axon use is evident at the time of hatching, several distinctive features of the adult motor patterns (Otis and Gilly, 1990) may emerge shortly thereafter. Figure 11A shows stellar nerve discharge of a 2-week-old *Septoteuthis* in response to an electric shock applied to the tentacles. A burst of small unit activity follows the stimulus at short latency; this is a pattern typical of embryonic and hatchling *Loligo*. The lower trace is plotted at an expanded time base. A stronger shock produced the response pictured in Figure 11B. A short latency, non-giant burst again occurs, but in this case it is followed by a giant axon spike after 80 ms. Timing of giant axon activation in relation to that of the small axons is adult-like (Fig. 5D), although the overall latency

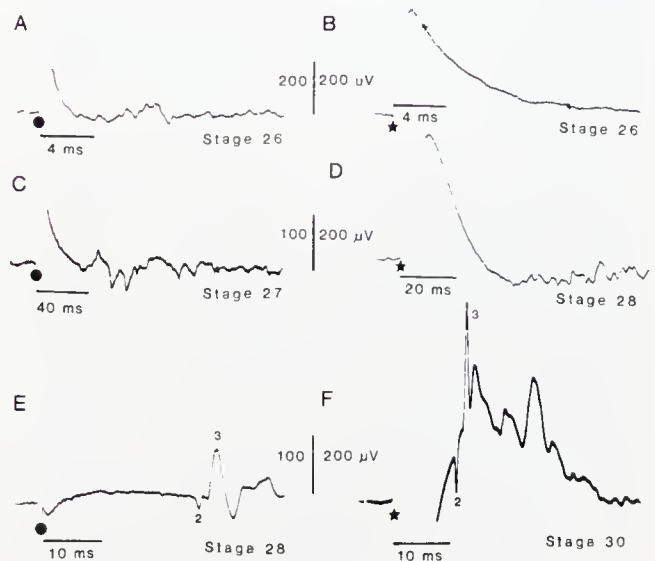


Figure 9. Functional maturation of the giant fiber pathway in embryonic *Loligo*. Indirect electrical (●) or light flash (*) stimuli were used to elicit escape responses at various stages of development; all recordings illustrated were obtained from the stellate ganglion. (A) At stage 26, electrical stimulation elicits only small axon activity. (B) A light flash is ineffective at stimulating escape responses or producing any detectable motor outputs at stage 26. (C) Giant axon activity is still not produced by electrical stimuli at stage 27. (D) Photic stimulation produces small axon activity but no giant spikes at stage 28. (E) Giant axon responses are first detectable at stage 28 with electrical stimuli. (F) A light flash stimulus at stage 30 produces a giant axon response. See text for additional details.

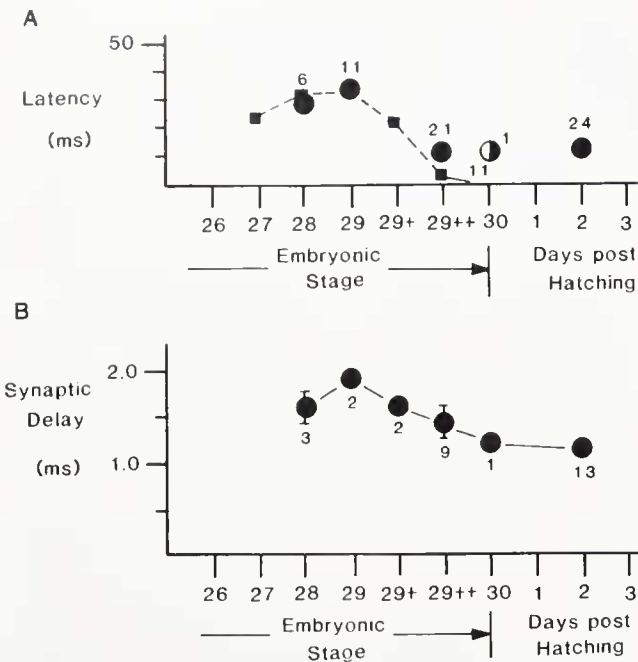


Figure 10. Improvement in performance of the giant fiber pathway during late embryogenesis in *Loligo*. (A) Response latency (time to second-order giant spike) decreases between stage 28 (time of first detectable giant axon activity) and 30 (hatching). Filled circles were obtained with indirect electrical stimuli; open symbol was obtained with light flashes. Mean values and the number of experiments are given; standard error of the mean is smaller than the symbols. Squares and dashed curve are behavioral data which has been replotted from Figure 3B. (B) Synaptic delay between the second- and third-order giants (across the giant synapse in the stellate ganglion) also decreases between stages 28 and 30. Means, number of experiments, and standard error are indicated.

is much less than that in adults (Fig. 5C). The motor pattern underlying the escape response in Figure 11B thus shows both embryonic and adult qualities.

More complex escape responses with the long delays characteristic of the adult can also be generated shortly after hatching in some animals. Results from another *Sepioteuthis* (~5 days post-hatching) are shown in Figure 12. Delayed escape jets driven by only the small axon system (Fig. 12A) and by both small and giant axons acting in concert (Fig. 12B) are well developed. Adult-like multiple cycle responses showing both of the above types of motor patterns are also evident (Fig. 12C). When the giant axons are used during such delayed escape responses, their firing is timed to occur after the non-giant system initiates the jet cycle. In every respect, the motor patterns in Figure 12 are adult-like. We do not yet know precisely when the adult-like motor patterns appear in *Sepioteuthis* after hatching, but stage 29–29++ embryos display only the fast-start embryonic pattern seen in *Loligo* (data not illustrated).

In addition to the delayed bouts of motor activity in Figure 12, an initial brief burst of activity occurs with a delay of ~20 ms in every case illustrated. The origin of

this activity is presently unknown, and recordings in adult squid also reveal similar short-latency activity in stellar nerves following an electrical stimulus (*cf.* Fig. 3 in Otis and Gilly, 1990). In the latter case, there is no detectable short-latency mantle contraction. Comparable behavioral experiments have not been carried out with juvenile *Sepioteuthis*.

Discussion

Motor control of escape behavior in late-stage embryos and hatchlings shows important similarities to the corresponding situation in adult squid. In both cases, escape jetting is under the influence of two parallel motor pathways: the giant fiber system and a small axon (non-giant) system. Strong escape jets can be driven by the small-axon system acting alone, with no giant fiber involvement whatsoever (Figs. 9C, 8A). The small axon pathway is the first to develop anatomically and become functional during embryonic development, and vigorous escape jets are possible before the giant axons appear.

Giant axon excitation provides a potent boost to the small axon system, however, and this greatly improves escape jetting performance. This was directly demonstrated in adult animals (Otis and Gilly, 1990). It is also evident in embryonic development (stages 28–30) as a marked decrease in response latency [seen behaviorally

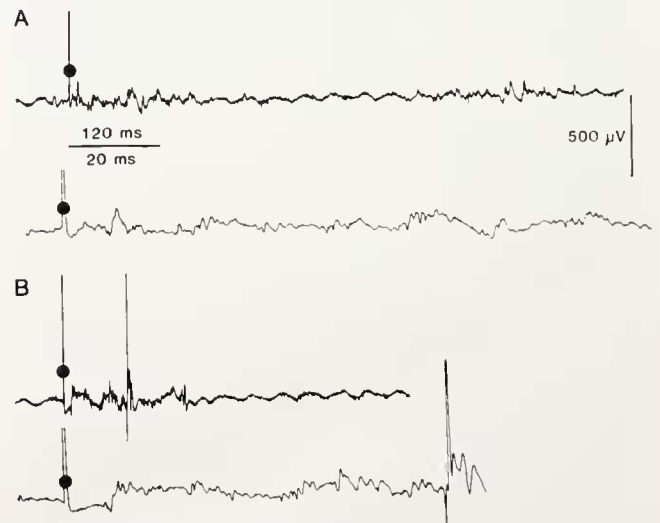


Figure 11. Partial development of adult-like use of the giant fiber pathway in a 14-day post-hatching *Sepioteuthis*. (A) Upper trace was recorded from the stellate ganglion following an electrical stimulus delivered to the base of the tentacles (●). Small unit activity only is generated, and the latency is brief. Lower trace shows the initial portion of the response following the shock displayed at an expanded time scale. (B) A stronger shock to the same site as in (A) produces a stronger burst of small axon activity at a short latency and a single giant axon spike in the middle of this burst. Lower trace shows the expanded version to identify the giant fiber spike. The occurrence of the giant spike well after the onset of the small unit burst is thus adult-like, but the brief latency (<20 ms) for the non-giant burst is characteristic of embryos.

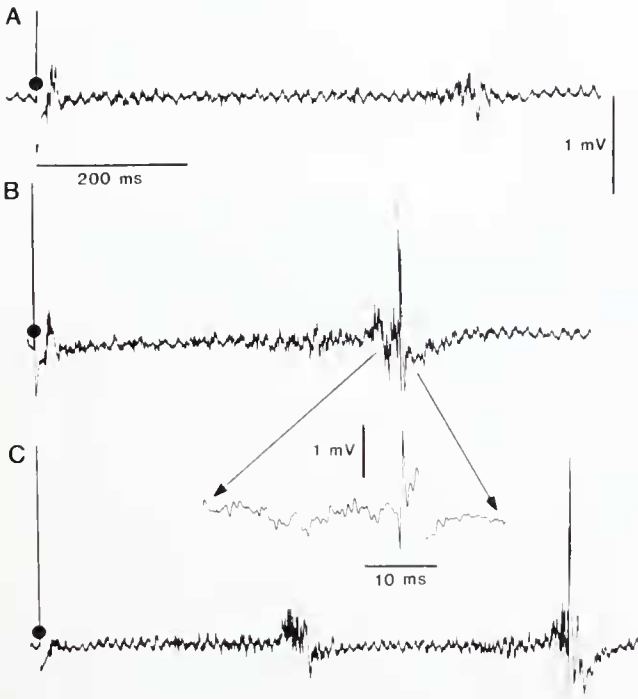


Figure 12. Fully developed adult-like interplay of giant and non-giant motor systems in escape responses of a 5-day post-hatching *Sepioteuthis*. (A) An electrical stimulus (●) to the side of the head produces an escape jet at a latency of ~ 500 ms that is driven only by small motor axons. (B) A stronger stimulus to the same site produces a delayed escape jet driven by both small axons and giant axons acting in concert. Non-giant activity precedes giant axon activation. Lower trace shows an expansion of the record around the giant fiber spike. (C) Stimulation on the ventral surface of the head just anterior to the eye produces a multiple cycle escape response at a long latency. The first cycle is driven by the small axon motor system acting alone, whereas the second also involves the giant axon pathway.

(Fig. 3B) or neurophysiologically (Fig. 10A)], time to peak mantle contraction (Fig. 3B), and rate of contraction (Fig. 3C). The time course of this improvement, beginning at stage 28–29, coincides closely with the anatomical appearance and maturation of the third-order giant motor axons in the more posterior stellar nerves that control jetting. Performance peaks at hatching. Presumably this is a valuable capability, because the embryonic squid must jet vigorously to escape from the confines of the egg mass in order to hatch.

At present it is not known how the neural portions of these giant and non-giant motor systems are associated with the two types of circular muscle fibers in adult squid mantle (Bone *et al.*, 1981; Mommsen *et al.*, 1981). Developing embryos and hatchlings would provide a valuable system in which to pursue this question, because the two motor systems do not develop in perfect synchrony. We have not carried out an anatomical analysis of the muscle fiber types in these young animals.

Although the overall picture of dual motor control of

escape jetting is similar in embryos, hatchlings, and adults, details of how the two motor systems are employed when they act together are strikingly different. In embryos and hatchlings, activity in the third-order giant axons always precedes the burst of small unit motor activity by several ms. This is true regardless of whether stimulation is by electric shock or light flash. Latency to firing for the giant axon varies from ~ 40 to 10 ms, depending on the exact stage of development (Fig. 10A). This delay basically represents the time required to excite the first-order giant cell (Fig. 7). Once this cell fires, the second- and third-order giants follow within 2–3 ms. In embryos and hatchlings, all escape responses are thus of the fast-start variety, although the latency is never so brief as with artificial direct stimulation of the first-order giant (Fig. 4).

In our experiments on adult squid, only sudden visual stimuli (*e.g.* light flash) have been effective in producing a fast-start pattern of giant axon excitation (Fig. 5A) like that seen in embryos or hatchlings, where giant axon firing precedes the burst of non-giant activity. Latency for giant axon activation, even in this fastest case, is much longer than that in hatchlings (50 ms *versus* 10 ms) and presumably again represents the time before the first-order giant fires.

Electrical stimuli in adults produce a second pattern of giant axon use that is not seen in the embryo. This delayed-escape mode shows a minimum latency of several hundred ms and, more significantly, a burst of non-giant motor output that commences 50–100 ms before the giant axons fire (Fig. 5C). The small-axon system thus appears to be the primary effector of this type of escape jet, and the giant axons are booster elements that are optionally recruited during any given cycle of a delayed-type response.

Otis and Gilly (1990) have argued that a great deal of complex processing in the central nervous system underlies effective use of the giant fiber pathway in this delayed-escape mode. This idea is in consonance with results on embryonic and hatchling *Loligo* presented in this paper. Much of the brain at these stages is not yet differentiated (Young, 1939; Martin, 1965), and these animals do not yet have the capability of orchestrating complex escape behavior, despite the functional presence of all the necessary peripheral motor components. Presumably, development of higher order neural centers is necessary to coordinate the interplay of giant and non-giant motor systems.

When and how does the strictly fast-start embryonic pattern in escape jetting evolve into the complex capabilities of the adult? This is not yet clear in *Loligo*. We have seen no indication of delayed escape responses in either behavioral or neurophysiological experiments for up to 6 days post-hatching. Preliminary work on post-hatching *Sepioteuthis* is also described in this paper. In this case, perfectly normal, adult-like firing patterns of the giant and non-giant motor systems were observed in an-

imals only 5 days old (Fig. 12), and other animals showed a curious mixture of embryonic and adult-like characteristics (Fig. 11).

The specimens of *Sepioteuthis* that we studied differed from our *Loligo* subjects in two important respects. First, *Sepioteuthis* is a much larger and possibly more developed (*i.e.*, neurologically) animal at birth. Second, the *Sepioteuthis* had been actively feeding and growing for the entire time after hatching (5–21 days), whereas *Loligo* was provided with no (for the neurophysiological work) or minimal food (for the behavioral work) and was maintained for no more than 6 days. Either or both of these factors could be relevant to the early attainment of coordinated delayed-escape jetting in young *Sepioteuthis*. In the first case, *Sepioteuthis* may simply develop the adult-like motor patterns more quickly after birth than does *Loligo*. The second possibility is more intriguing, however.

Hatchling squid have limited energy reserves sufficient only for several days (O'Dor *et al.*, 1986) during which time the animals must learn to capture prey or face starvation (Hurley, 1976; Yang *et al.*, 1983, 1986). Because prey items consist largely of fast-moving copepods, and high speed pursuit is involved in prey-capture, it would seem advantageous to employ the giant fiber pathway in this activity. To do this effectively, however, strict control over excitation of the first order giants must be necessary to provide the giant axon-mediated boost precisely at the correct moment. This capability—critically timed excitation of the giant fiber pathway—is the basic feature of the adult delayed-escape response that differentiates it from the fast-start response. The possibility that such a profound change-over in the pattern of giant axon use in hatchling squid might be associated with the perfection of feeding behavior is a prospect that we are currently pursuing.

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